METHOD OF INHIBITING REJECTION FOLLOWING ORGAN TRANSPLANTATION

CROSS-REFERENCE

[001] The present application claims benefit under 35 USC 119(e) of the U.S. provisional application No. 60/515,254 filed on October 29, 2003, the contents of which are incorporated herein by reference in their entirety.

GOVERNMENT SUPPORT

[002] This invention was made with Government Support under Contract Number AI46756 awarded by the National Institutes of Health. The Government has certain rights in the invention.

BACKGROUND OF THE INVENTION

[003] Renal transplantation is the definitive therapy for chronic renal failure and has improved the quality of and prolonged the life of thousands since its inception over three decades ago. Despite significant advances in understanding of tissue typing and immunosuppression and the availability of better immunosuppressive agents, rejection remains a serious clinical problem. In the absence of successful therapies, rejection will lead to graft failure in some patients, requiring reinstitution of dialysis and the search for another donor kidney. With the use of cyclosporine in conjunction with other immunosuppressive agents, the one-year graft survival rate for cadaver allografts is in the range of 80%, but graft half-life remains less than optimal in the range of 7.2 years. Kirkman et al., Transplantation, 1991:51:107-113. Addition of new immunosuppressive agents and Mycophenolate Moefitil and/or FK506 have further improved 1 year graft survival to over 90% . Other types of solid organ transplantation, e.g., heart, liver and lung, also save the lives of thousands of patients each year, but here also rejection remains a serious clinical problem only partially controlled by current immunosuppressive drugs.

[004] Current immunosuppressive therapy for rejection associated with renal or other solid organ transplantation consists of multiple drugs that interfere with the function of the immune system at various levels. In addition to the complications of over-immunosuppression that may result from the use of multiple drugs, each has its own unique toxicity profile, which may limit its usefulness.

[005] It is well established that leukocyte recruitment into sites of inflammation is associated with angiogenesis and there is considerable evidence to suggest that angiogenesis and inflammation occur in an interactive and overlapping manner (1-4). Vascular Endothelial Growth Factor (VEGF) is a potent angiogenesis factor that has important roles in both normal physiological as well as pathological vasculogenesis and angiogenesis (5-7). VEGF stimulates endothelial cell proliferation and migration in vitro, and has also been found to reprogram endothelial cell gene expression and to preserve endothelial cells from undergoing apoptosis and senescence (7;8). In addition, VEGF acts as a proinflammatory cytokine by increasing endothelial cell permeability, inducing the expression of endothelial cell adhesion molecules and via its ability as a monocyte chemoattractant (9-11). Thus, VEGF is likely a key intermediary between cell-mediated immune inflammation and the associated angiogenesis reaction.

[006] VEGF is produced by endothelial cells, macrophages, activated T cells and a variety of other cell types (7;12;13). It is a heparin-binding, homodimeric glycoprotein with several protein variants resulting from alternative mRNA splicing (5). VEGF binds to three high affinity tyrosine kinase receptors Flt-1 (VEGFR-1), KDR (VEGFR-2) (7) and neuropilin (14) expressed almost exclusively by endothelial cells; although Flt-1 is also expressed by monocytes (15). The major stimulus for VEGF expression is hypoxia (16), but other factors that can upregulate VEGF expression include the degree of cell differentiation, local concentrations of glucose and serum, hormones, prostaglandins, modulators of protein kinase C, nitric oxide and stimulators of adenylate cyclase (7;17-20). In addition, we recently demonstrated that ligation of CD40 on endothelial cells and monocytes by CD40 ligand (expressed on platelets and activated T cells) is potent for the induction of VEGF, thereby linking immune inflammation with angiogenesis (12;21).

[007] A need exists for improved therapeutic methods of inhibiting graft/organ rejection.

SUMMARY OF THE INVENTION

[008] The present invention is directed to methods for inhibiting organ/graft rejection. In one embodiment, the method of the present invention

comprises administering to a donor, prior to transfer of donor tissue, an effective amount of VEGF antagonist. In an alternative of this embodiment, as part of the same protocol, a VEGF antagonist is also administered to the organ recipient. The VEGF antagonist can be the same or different than that administered to the organ donor. As another alternative to the embodiment, as part of the transplant therapeutic protocol, the same VEGF antagonist is administered to the donor prior to the transplant and to the recipient after the transplant. An immunosuppressant and/or chemokine antagonist may also be administered to the organ donor and/or the recipient.

[009] In one embodiment of the present invention, the donor is a marginal donor and the donor tissue is marginal tissue. The terms "marginal donor(s)" and "marginal tissue" are used herein as general terms to describe a donor or tissue presenting with problems that render its use in a transplantation procedure less than optimal. For example, a marginal donor can include a donor that is older than 50 years old, or that is afflicted with a chronic disease that may affect graft function, e.g., diabetes, hypertension and alcohol intake. A marginal tissue is, for example, (1) an organ from such a donor, or (2) an organ that has experienced prolonged warm or cold ischemia times, or (3) an organ that presents with anatomical abnormalities (e.g., small and multiple vessels, e.g., in the kidney) that can render the vascular anastomosis difficult, or with evidence of atherosclerostic plaques on graft vessels.

[0010] Alternatively, the donor is non-heartbeating donor (NHBD). A non-heartbeating donor is a donor whose heart has stopped beating and all resuscitation methods have failed.

[0011] In another embodiment, the method comprises administering to an organ transplant recipient and/or to a donor animal an effective amount of an anti-VEGF antibody an immunosuppressant and/or chemokine antagonist.

[0012] As used herein, "inhibiting rejection" includes treatment or prevention transplant rejection (e.g. , acute or chronic graft rejection).

[0013] The term VEGF antagonists includes agents that decrease, inhibit, block or interfere with VEGF function including VEGF binding to the VEGF receptor KDR (VEGFR-2), VEGF receptor signaling and VEGF production. (*See,* WO

963004; WO 0037502 and Jayson et al., Expert opinion on Biological Therapy 1:703 (2001)). Such antagonists include, for example, antibodies as defined herein, and molecules having antibody-like function such as synthetic analogues of antibodies, e.g., single-chain antigen binding molecules, small binding peptides, or mixtures thereof. Compounds having antagonist activity also include small organic molecules, natural products, peptides, aptamers, peptidomimetics, siRNA, ribozymes, and RNAi.

[0014] An antibody is a preferred VEGF antagonist. Preferably, the antibody is directed against the N-terminal sequence of secreted VEGF. Preferably, the antibody is directed against the N-terminal 25 amino acids. Most preferably, the antibody is directed against the N-terminal 20, 21, 22, 23, 24, or 25 amino acids. A preferred anti-VEGF antibody is a humanized monclonal antibody, e.g., Bevacizamab (Avastin™, Genetech). Alternatively, the anti-VEGF antibody is IMC-1C11 (ImClone Systems), DC 101 (a KDR VEGF Receptor 2 from ImClone Systems), or a humanized 2G11 antibody as described in Example 3.

[0015] Alternatively, the VEGF inhibitor is a low molecular weight compound such as SU-6668, SU-5416 (Cancer Res., 60, 4152 - 4160 (2000)), or ZK222584 (Cancer Res., 60, 2178 - 2189 (2000)). Preferably the inhibitor is PTK787 (Novartis Pharmaceuticals), a VEGF receptor signaling inhibitor,.

[0016] Other VEGF inhibitors are described in, for example in WO 99/24440 (published May 20, 1999), International Application PCT/IB99/00797 (filed May 3, 1999), in WO 95/21613 (published August 17, 1995), WO 99/61422 (published December 2, 1999), United States Patent 5,834,504 (issued November 10, 1998), WO 98/50356 (published November 12, 1998), United States Patent 5,883,113 (issued March 16, 1999), United States Patent 5,886,020 (issued March 23, 1999), United States Patent 5, 792,783 (issued August 11, 1998), WO 99/10349 (published March 4, 1999), WO 97/32856 (published September 12, 1997), WO 97/22596 (published June 26, 1997), WO 98/54093 (published December 3, 1998), WO 98/02438 (published January 22, 1998), WO 99/16755 (published April 8, 1999), and WO 98/02437 (published January 22, 1998), all of which are herein incorporated by reference in their entirety.

[0017] Other examples of specific VEGF inhibitors are IM862 (Cytran Inc. of Kirkland, Washington, USA), rapamycin and Angiozyme (Sirna Therapeutics),

a synthetic ribozyme which specifically recognizes the mRNA for FLT-1, one of the most important VEGF receptors in angiogenesis. Unlike normal drugs, which interfere with the activity of the target protein itself, ribozyme drugs destroy the mRNA and prevent the protein being synthesized in the first place.

[0018] Anti-VEGF receptor 2 inhibitory compounds may also be used and include anti-KDR antibodies and functional equivalents thereof, including monoclonal antibodies (USP 6,121,230). Functional equivalents include, for example, chimerized, humanized and single chain antibodies and fragments thereof.

[0019] Antisense oligonucleotides may also be used as anti-VEGF receptors 2 inhibitory compounds. Preferably, the antisense oligonucleotides derived from the translation initiation site, e.g., between -10 and +10 regions of the Flk-1 nucleotide sequence, are preferred. See, USP 5,851,999.

[0020] Ribozmes, iRNA and soluble FLK-1 (USP 5,851,999) may also be used.

Preferably, the compound is a small molecule inhibitor of Flk-1/KDR such as those disclosed in USP 5,849,742. Preferred include, for example, the di-t-butly phenol analogs SU 0879, SU 1498 (typhostin) and SU 1433, all available from Sugen.

[0021] The method of the present invention is useful in the treatment and prevention of transplant rejection. More specifically, the method may be employed for the treatment of a patient that has undergone organ transplantation that is either allogeneic or xenogeneic. Furthermore, the method of the present invention may be utilized prior to, following or concurrently with the transplant procedure, or any combination thereof.

[0022] Preferred immunosuppressants include cyclosporine (Sandimmune®, Neoral®), FK-506, rapamycin, corticosteroids, cyclophosphamide, mycophenolate mofetil (MMF) (Cellcept®), Myfortic (mycophenolate sodium, Novartis), leflunomide, anti-lymphocyte globulin, deoxyspergualin OKT-3 and the like. Cyclosporin and MMF are more preferred.

[0023] The term "antibody", includes human and animal mAbs, and preparations of polyclonal antibodies, as well as antibody fragments, synthetic

antibodies, including recombinant antibodies (antisera), chimeric antibodies, including humanized antibodies, anti-idiotopic antibodies and derivatives thereof.

[0024] The term "chemokine (receptor) antagonist" means any molecule that can inhibit activity of a chemokine. Antagonists of the chemokine receptor CXCR3 are preferred. (*See,* WO 01/78708 for a disclosure of such antagonists. Other chemokine receptors include CCR1, CCR2, CCR3, CCR4, CCR5, CCR6, CCR7, CCR8, CCR9 and CCR10.

BRIEF DESCRIPTION OF THE FIGURES

[0025] Figure 1: Anti-VEGF anti-serum neutralizes VEGF *in vivo*. VEGF neutralizing activity of anti-serum was assessed *in vivo* using a modified version of a standard VEGF-induced angiogenesis assay. Briefly, Chinese Hamster Ovary (CHO) cells (1×10^5 cells) expressing VEGF were injected into the ears of nude mice. The mice received control serum or anti-VEGF antiserum (0.8mls) i.p. daily starting at day –1. Marked angiogenesis was evident at day 2-4 in control serum-treated mice. Overall, using this *in vivo* assay, there was ~80% inhibition of VEGF-induced angiogenesis by the anti-serum.

[0026] Figure 2: Expression of VEGF in association with human leukocytic infiltration of skin. SCID mice bearing healed human skin transplants received 3×10^8 human PBL or saline by intraperitoneal injection. Skin grafts were harvested after 14 days. Figures 2A and 2B, the expression of VEGF mRNA in normal non-infiltrated skins (N, non-humanized SCID) or in infiltrated skin specimens (I, huSCID) evaluated by RT-PCR and by RNase protection assay respectively. Figure 2C, the relative expression of VEGF mRNA vs. GAPDH evaluated by RNase protection quantified by densitometry. Bar graphs illustrate mean VEGF expression (+ 1 SD) for three non infiltrated (N) or infiltrated (I) skins. Figures 2D and 2E, expression of VEGF (red) by immunohistochemistry in an infiltrated skin specimen. Figure 2F, expression of VEGF (red) in a normal non-infiltrated skin. Note that there is enhanced VEGF expression in association with leukocytic infiltration. Representative of at least 10 experiments. (Magnification of Panels d-f 400x).

[0027] Figure 3: Blockade of VEGF inhibits human leukocyte recruitment. huSCID mice bearing human skin transplants received saline, IgG, or anti-human VEGF. Skin grafts were harvested 14 days after humanization and infiltrates were identified by H&E staining (Figures 3A – 3C), by immunostaining with anti-human CD3 (Figures 3D – 3F) and by immunostaining with anti-human CD68 (Figures 3G – 3I). Treatment of huSCID with anti-human VEGF inhibited both CD3+ T cell (Figure 3F) and CD68+ monocyte macrophage (Figure 3I) infiltration of skin. Figure 3J, quantitative assessment of CD3+ T cell infiltrates was performed by calibrated grid counting at 400x magnification in skin specimens harvested at either day 7 or day 14 following humanization. The mean CD3 count per calibrated field is illustrated in skins harvested from animals treated with either saline (n=7 at day 7; n=10 at day 14), anti-human VEGF (n=5 at day 7; n=10 at day 14), or control IgG (n=4 at day 14).

[0028] Figure 4: Effect of VEGF on endothelial cell chemokine expression. Confluent cultures of human endothelial cells were treated for 4 hrs (Figures 4A and 4C) or as a time course (Figures 4B and 4D) with VEGF alone or VEGF in combination with IFN-γ as indicated. Total RNA was harvested from endothelial cells and the expression of chemokines was analyzed by RNase protection assay. a, concentration, and b, time-dependent effects of VEGF on chemokine expression. c and d, effect of IFN-γ alone or in combination with VEGF on chemokine expression. Note that treatment with IFN-γ alone resulted in IP-10 expression (Figure 4C, and Figure 4D - Lane 7), and a combination of IFN-γ with VEGF resulted in a synergistic induction of IP-10 (Figure 4C, and Figure 4D - Lanes 2-6). Bar graphs to the right of each blot represent the quantitative analysis of IP-10 mRNA expression in 3 representative RNase protection assays as illustrated in Figures 4C and 4D. Figure 4E, the production of IP-10 by ELISA in culture supernatants of IFN-γ (1000U/ml, ■)- or VEGF (10ng/ml) and IFN-γ ((1000U/ml, ❑)- treated endothelial cells for different times as indicated. Representative of 3 similar experiments performed in triplicate (mean + 1 SD).

[0029] Figure 5: Blockade of VEGF in a fully mismatched murine model of acute cardiac allograft rejection. Fully mismatched C57BL/6 (H-2b) donor hearts were transplanted into BALB/c (H-2d) mice as recipients. 5Untreated recipients

develop marked leukocytic infiltrates and rejection by day 7. Figures 5A and 5B, immunohistochemical analysis of VEGF in a normal non-transplanted heart and in a day 7 rejecting allograft respectively, showing intense VEGF expression in association with allograft rejection. Figure 5C, graft survival curves for recipients treated with anti-VEGF antiserum (■n=6) or normal rabbit serum as a control (❑ n=5). Anti-VEGF-treated recipients show prolonged heart allograft survival ($P<0.001$). d-m, histological analysis of cardiac allografts harvested at day 7 post-transplantation from rejecting control serum-treated animals (Figures 5D – 5H) or from animals treated with anti-VEGF (i-m). Control serum treated animals had evidence of severe cellular rejection with extensive mononuclear cell infiltration (Figure 5D) including CD45+ (Figure 5E) CD3+ (Figure 5F), and macrophage (Figure 5G) infiltrates. In contrast, recipients treated with anti-VEGF had minimal infiltrates and no evidence of vasculitis (Figures 5I – 5L). Figure 5H and 5M, IP-10 protein expression was diffuse and intense within allografts in association with rejection (Figure 5H) but was of low intensity and sparse in anti-VEGF treated recipients (Figure 5M). Representative immunostaining of 5 animals from each group, magnification 400x.

[0030] Figure 6: Function of VEGF in alloimmune T cell activation and allograft rejection. Figure 6A, anti-human VEGF or anti-murine VEGF antiserum was added into the human or the mouse MLR respectively. Proliferation was assessed by 3[H] thymidine incorporation for the last 18 hrs of co-culture. Figure 6B, the production of interferon-γ and IL-2 was assessed by ELISA in coculture supernatants from a human MLR. As illustrated, blockade of VEGF had no effect on proliferation or cytokine production in the MLR. (bars indicate the mean ± 1 SD for triplicate wells). Data are representative of 3 experiments with similar results. Figure 6C, frequency of IFN-γ producing cells in murine recipients of cardiac transplants as assessed by ELISPOT. Illustrated is the production of IFN-γ from a syngeneic, an untreated and an anti-VEGF-treated animal. Representative of three such experiments performed in triplicate.

[0031] Figure 7: Function of Angiogenesis in Acute Rejection: Fully mismatched C57BL/6 (H-2b) donor hearts were transplanted into BALB/c (H-2d) mice; and recipients were treated with control Ig, anti-VEGF or endostatin as

described in Methods. Figure 7A, Immunohistochemical analysis of CD31 in isografts or allografts harvested at day 7 from animals treated with control Ig or anti-VEGF. Figure 7B, H&E staining of allografts harvested from day 7 untreated or endostatin-treated animals showing marked infiltrates in both untreated and treated grafts at low magnification (left, 200x) and at high magnification (right, 400x). Representative of 8 animals. Figure 7C, Immunostaining for CD3-expressing T cells in a representative allograft from an endostatin-treated animal; Figure 7D, graft survival curves for untreated recipients (dotted line, n=10), or recipients treated with endostatin (solid line, n=8). Note that the endostatin used in these studies inhibited angiogenesis in control animals with tumors (as described (30)), but only minimally prolonged graft survival in three of 8 animals.

[0032] Figure 8: Function of VEGF-dependent regulation of IP-10 in allograft rejection. Figure 8A: Confluent cultures of murine myocardial endothelial cells were treated for 4 hrs with recombinant murine VEGF or IFN-γ alone or VEGF in combination with IFN-y as indicated. Total RNA was harvested from endothelial cells and the expression of IP-10 was analyzed by RNase protection assay. Figures 8B and 8C: The ability of VEGF to mediate IP-10-dependent trafficking and rejection was evaluated using anti-VEGF and anti-IP-10 in fully mismatched C57BL/6 (H-2b) or IP-10 -/- (H-2b) donor hearts transplanted into BALB/c (H-2d) mice. Recipients of wild type grafts were treated with anti-VEGF alone, or with anti-VEGF in combination with anti-IP-10. Recipients of IP-10 -/- donor grafts were treated with anti-VEGF. Both anti-IP-10 and anti-VEGF were administered according to the schedule outlined in Methods. As illustrated in Figure 8B, we found that addition of anti-VEGF with anti-IP-10 significantly prolonged allograft survival in wild type combinations ($P<0.005$); and in Figure 8C, anti-VEGF prolonged survival in mice who received IP-10-/- donor hearts ($P<0.04$). The survival of control untreated wild-type grafts are illustrated as the dotted line.

[0033] Figure 9: Effect of Anti-VEGF on the intragraft expression of adhesion molecules and chemokines. Analysis of endothelial cell adhesion molecule expression (Figure 9A) and chemokine expression (Figure 9B) in day 7 cardiac syngeneic grafts (Syn) or allografts (Allo). Recipients received either control normal rabbit serum (-) or anti-VEGF antiserum (+). The bar graphs represent quantitative

analysis of mRNA expression as the relative expression of adhesion molecules (adh) or chemokine (chem) compared to the expression of GAPDH. Mean expression (+1 SD) for 3 animals treated with normal rabbit serum animals (open bars) or anti-VEGF antiserum (solid bars) is shown.

[0034] Figure 10: The effect of Cyclosporine (CsA) in combination with VEGF on graft survival. CsA is a well established immunosuppressant that inhibits T cell activation. It has been found to inhibit and to stimulate the production of VEGF depending on the cell type examined. Here, we have analyzed the physiological relevance of the interaction between VEGF and CsA for allograft survival *in vivo*. Fully mismatched C57BL/6 (H-2b) donor hearts were transplanted into BALB/c (H-2d) mice; and recipients were treated with CsA alone(10mg/Kg daily by i.p. injection for 14 days), anti-VEGF alone (0.8 mls every other day for 6 days by i.p injection as described above) or CsA in combination with anti-VEGF. Figure 10A, Mean graft survival (+ 1 SEM) for untreated recipients (N=10), recipients treated with CsA alone (N=8) or VEGF alone (N=8); or recipients treated with both agents (N=8). Figure 10B: Graft survival curves for the same groups of animals illustrating that treatment with CsA (l) or anti-VEGF (s) alone prolonged allograft survival above untreated controls (dotted line); and the combination of CsA with anti-VEGF (n) prolonged survival significantly compared to each agent alone ($P<0.001$ for CsA + Anti-VEGF vs. CsA alone; $P<0.03$ for CsA + anti-VEGF vs. Anti-VEGF alone).

[0035] Figure 11: Anti-VEGF mAB 2G11 neutralizes VEGF in vivo. VEGF neutralizing activity of 2G11 was assessed in vivo using a modified version of a standard VEGF-induced angiogenesis assay. Briefly, Chinese Hamster Ovary (CHO) cells (1×10^5 cells) expressing VEGF were injected into the ears of nude mice. This results in a most potent and aggressive angiogenesis reaction. The mice received either no treatment or 2G11 anti-VEGF in increasing doses i.p. daily starting at day –1. Figure 11 A, is seen marked angiogenesis in response to CHO-VEGF cell injection at day 2, whereas in figure 11B, the antibody at a dose of 200mcg/day inhibits this CHO-VEGF induced angiogenesis reaction. This clearly indicates that 2G11 blocks VEGF function in vivo. (Same result is seen at day 4, not shown).

[0036] Figure 12: Ischemia Reperfusion Injury (Kidney): Unilateral acute tubular necrosis (ATN) in mice by clamping of the renal pedicle to produce

ischemia. Ischemic injury occurs using 30 minute timed protocols, after which the clamps are removed. Animals are monitored daily. Tissue is harvested at various time points after surgery for up to 4 weeks. Graft function is monitored by measurement of serum creatinine every few hrs for first 24hrs, then daily or every other day. Treatment is PTK 787 at a dose of 4mg/day and mycophenolate moefitil (MMF) at 2mg/day

[0037] Figure 13: Serum alanine aminotransferase (ALT). The liver model is described in the reference (Transplantation, 2003 April 27: 75; pages 1118-1123) and is similar to the kidney model described in Example 4. Animals were treated with the anti-VEGF anti-serum (0.8 ml intraperitoneally) on Day –1 and at the time of ischemia. Control antibody treated animals developed a marked rise in serum transaminase levels (ALT) with ALT a mean level of 3,619 + 750. In contrast, anti-VEGF treated animals only developed a mild increase in transaminase levels of 413 + 126. (The rise in ALT transaminase is indicative of liver injury. Normal levels are less than 100.) These results suggest that the blockade of VEGF has protective effects in the prevention of ischemia reperfusion injury in the liver.

DETAILED DESCRIPTION OF THE INVENTION

[0038] A first aspect of the invention provides a method for inhibiting rejection of a graft organ, comprising administering to a donor an effective amount of a VEGF antagonist before the organ is harvested. The VEGF antagonist is preferably administered at least 24 hours before the organ is harvested. As part of the same protocol the organ recipient is administered a VEGF antagonist. The VEGF antagonist may be administered to the recipient before, at the time of and/or after transplant.

[0039] Preferably, the VEGF or chemokine antagonist function is a compound which is, for example, a small organic molecule, natural product, protein (e.g., antibody, chemokine, cytokine), peptide or peptidomimetic. Antagonists can be identified, for example, by screening libraries or collections of molecules, such as, the Chemical Repository of the National Cancer Institute, as described herein or using other suitable methods.

[0040] Another source of antagonists is combinatorial libraries which can comprise many structurally distinct molecular species. Combinatorial libraries can be used to identify lead compounds or to optimize a previously identified lead. Such libraries can be manufactured by well-known methods of combinatorial chemistry and screened by suitable methods, such as the methods described herein.

[0041] The term "peptide", as used herein, refers to a compound consisting of from about two to about ninety amino acid residues wherein the amino group of one amino acid is linked to the carboxyl group of another amino acid by a peptide bond.

[0042] A peptide can be, for example, derived or removed from a native protein by enzymatic or chemical cleavage, or can be prepared using conventional peptide synthesis techniques (e.g., solid phase synthesis) or molecular biology techniques (see Sambrook, J. et al., Molecular Cloning : A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, NY (1989)). A "peptide" can comprise any suitable L-and/or D-amino acid, for example, common a-amino acids (e.g., alanine, glycine, valine), non-a-amino acids (e.g., P-alanine, 4-aminobutyric acid, 6aminocaproic acid, sarcosine, statine), and unusual amino acids (e.g., citrulline, homocitruline, homoserine, norleucine, norvaline, ornithine). The amino, carboxyl and/or other functional groups on a peptide can be free (e.g., unmodified) or protected with a suitable protecting group. Suitable protecting groups for amino and carboxyl groups, and means for adding or removing protecting groups are known in the art and are disclosed in, for example, Green and Wuts, "Protecting Groups in Organic Synthesis", John Wiley and Sons, 1991. The functional groups of a peptide can also be derivatized (e.g., alkylated) using art-known methods.

[0043] Peptides can be synthesized and assembled into libraries comprising a few to many discrete molecular species. Such libraries can be prepared using well-known methods of combinatorial chemistry, and can be screened as described herein or using other suitable methods to determine if the library comprises peptides which can antagonize VEGF function. Such peptide antagonists can then be isolated by suitable methods.

[0044] The term "peptidomimetic", as used herein, refers to molecules which are not polypeptides, but which mimic aspects of their structures. For example,

polysaccharides can be prepared that have the same functional groups as peptides which can antagonize VEGF. Peptidomimetics can be designed, for example, by establishing the three dimensional structure of a peptide agent in the environment in which it is bound or will bind to VEGF. The peptidomimetic comprises at least two components, the binding moiety or moieties and the backbone or supporting structure.

[0045] The binding moieties are the chemical atoms or groups which will react or form a complex (e.g., through hydrophobic or ionic interactions) with VEGF, for example, with the amino acid (s) at or near the ligand binding site. For example, the binding moieties in a peptidomimetic can be the same as those in a peptide antagonist of VEGF. The binding moieties can be an atom or chemical group which reacts with the receptor in the same or similar manner as the binding moiety in a peptide antagonist of VEGF. Examples of binding moieties suitable for use in designing a peptidomimetic for a basic amino acid in a peptide are nitrogen containing groups, such as amines, ammoniums, guanidines and amides or phosphoniums. Examples of binding moieties suitable for use in designing a peptidomimetic for an acidic amino acid can be, for example, carboxyl, lower alkyl carboxylic acid ester, sulfonic acid, a lower alkyl sulfonic acid ester or a phosphorous acid or ester thereof.

[0046] The supporting structure is the chemical entity that, when bound to the binding moiety or moieties, provides the three dimensional configuration of the peptidomimetic. The supporting structure can be organic or inorganic. Examples of organic supporting structures include polysaccharides, polymers or oligomers of organic synthetic polymers (such as, polyvinyl alcohol or polylactide). It is preferred that the supporting structure possess substantially the same size and dimensions as the peptide backbone or supporting structure. This can be determined by calculating or measuring the size of the atoms and bonds of the peptide and peptidomimetic. In one embodiment, the nitrogen of the peptide bond can be substituted with oxygen or sulfur, thereby forming a polyester backbone. In another embodiment, the carbonyl can be substituted with a sulfonyl group or sulfinyl group, thereby forming a polyamide (e.g., a polysulfonamide). Reverse amides of the peptide can be made (e.g., substituting one or more-CONH-groups for a-NHCO-group). In yet another embodiment, the peptide backbone can be substituted with a polysilane backbone.

[0047] These compounds can be manufactured by known methods. For example, a polyester peptidomimetic can be prepared by substituting a hydroxyl group for the corresponding a-amino group on amino acids, thereby preparing a hydroxyacid and sequentially esterifying the hydroxyacids, optionally blocking the basic and acidic side chains to minimize side reactions. An appropriate chemical synthesis route can generally be readily identified upon determining the desired chemical structure of the peptidomimetic.

[0048] Peptidomimetics can be synthesized and assembled into libraries comprising a few to many discrete molecular species. Such libraries can be prepared using well known methods of combinatorial chemistry, and can be screened as described herein to determine if the library comprises one or more peptidomimetics which antagonize VEGF function. Such peptidomimetic antagonists can then be isolated by suitable methods.

[0049] As used herein, an "antibody that inhibits VEGF activity" or "anti-VEGF antibody" includes an antibody or antigen-binding fragment. The antibody can be polyclonal or monoclonal, and the term "antibody" is intended to encompass both polyclonal and monoclonal antibodies. The terms polyclonal and monoclonal refer to the degree of homogeneity of an antibody preparation, and are not intended to be limited to particular methods of production. The term "antibody" as used herein also encompasses functional fragments of antibodies, including fragments of chimeric, human, humanized, primatized, veneered or single-chain antibodies. Functional fragments include antigen-binding fragments which bind to VEGF or KDR. For example, antibody fragments capable of binding to VEGF or portions thereof, including, but not limited to Fv, Fab, Fab'and F (ab') 2 fragments can be used. Such fragments can be produced by enzymatic cleavage or by recombinant techniques. For example, papain or pepsin cleavage can generate Fab or F (ab') 2 fragments, respectively. Other proteases with the requisite substrate specificity can also be used to generate Fab or F (ab') 2 fragments. Antibodies can also be produced in a variety of truncated forms using antibody genes in which one or more stop codons have been introduced upstream of the natural stop site. For example, a chimeric gene encoding a F (ab') 2 heavy chain portion can be designed to include DNA sequences encoding the CH, domain and hinge region of the heavy chain. Single-chain antibodies, and

chimeric, human, humanized or primatized (CDR-grafted), or veneered antibodies, as well as chimeric, CDR-grafted or veneered single-chain antibodies, comprising portions derived from different species, and the like are also encompassed by the present invention and the term "antibody". The various portions of these antibodies can be joined together chemically by conventional techniques, or can be prepared as a contiguous protein using genetic engineering techniques. For example, nucleic acids encoding a chimeric or humanized chain can be expressed to produce a contiguous protein. See, e.g., Cabilly et al., U. S. Patent No. 4, 816, 567 ; Cabilly et al., European Patent No. 0,125,023 B1; Boss et al., U. S. Patent No. 4,816,397; Boss et al., European Patent No. 0,120,694 B1; Neuberger, M. S. et al., WO 86/01533; Neuberger, M. S. et al., European Patent No. 0,194,276 B1; Winter, U. S. Patent No. 5,225,539; Winter, European Patent No. 0,239,400 B1; Queen et al., European Patent No. 0451216 B1 ; and Padlan, E. A. et al., EP 0519596 A1. See also, Newman, R. et al., BioTechnology, 10: 1455-1460 (1992), regarding primatized antibody, and Ladner et al., U. S. Patent No. 4,946,778 and Bird, R. E. et al., Science, 242: 423-426 (1988)) regarding single-chain antibodies.

[0050] Humanized antibodies can be produced using synthetic or recombinant DNA technology using standard methods or other suitable techniques. Nucleic acid (e.g., cDNA) sequences coding for humanized variable regions can also be constructed using PCR mutagenesis methods to alter DNA sequences encoding a human or humanized chain, such as a DNA template from a previously humanized variable region (see e.g., Kamman, M., et al., Nucl. Acids Res., 17: 5404 (1989)); Sato, K., et al., Cancer Research, 53: 851-856 (1993); Daugherty, B. L. et al., Nucleic Acids Res., 19 (9): 2471-2476 (1991); and Lewis, A. P. and J. S. Crowe, Gene, 101: 297-302 (1991)). Using these or other suitable methods, variants can also be readily produced. In one embodiment, cloned variable regions can be mutated, and sequences encoding variants with the desired specificity can be selected (e.g., from a phage library; see e.g., Krebber et al., U. S. 5,514,548 ; Hoogenboom et al., WO 93/06213, published April 1, 1993).

[0051] Antibodies which are specific for mammalian (e.g., human) VEGF can be raised against an appropriate immunogen, such as isolated and/or recombinant human VEGF or portions thereof (including synthetic molecules, such as synthetic

peptides). The N-terminal protion of VEGF is preferred. SEQ ID NO:1 is a preferred portion.

[0052] Alternatively, a useful antibody of the present invention is a humanized anti- mouse VEGF monoclonal antibody. For example, rat anti-mouse VEGF monoclonal antibody 2G11 (See Example 3) may be humanized using techniques known to those of skill in the art, (see e.g., Kamman, M., et al., Nucl. Acids Res., 17: 5404 (1989)); Sato, K., et al., Cancer Research, 53: 851-856 (1993); Daugherty, B. L. et al., Nucleic Acids Res., 19 (9): 2471-2476 (1991); and Lewis, A. P. and J. S. Crowe, Gene, 101: 297-302 (1991)), and used in the methods of the present invention.

[0053] Preparation of immunizing antigen, and polyclonal and monoclonal antibody production can be performed using any suitable technique. A variety of methods have been described (see e.g., Kohler et al., Nature, 256 : 495-497 (1975) and Eur. J. linmunol. 6: 511-519 (1976); Milstein et al., Nature 266: 550-552 (1977); Koprowski et al., U. S. Patent No. 4,172,124; Harlow, E. and D. Lane, 1988, Antibodies: A Laboratory Manual, (Cold Spring Harbor Laboratory : Cold Spring Harbor, NY); Current Protocols In Molecular Biology, Vol. 2 (Supplement 27, Summer '94), Ausubel, F. M. et al., Eds., (John Wiley & Sons: New York, NY), Chapter 11, (1991)). When a monoclonal antibody is desired, a hybridoma can generally be produced by fusing a suitable immortal cell line (e.g., a myeloma cell line such as SP2/0 or P3X63Ag8. 653) with antibody-producing cells. The antibody producing cells, preferably those obtained from the spleen or lymph nodes, can be obtained from animals immunized with the antigen of interest. The fused cells (hybridomas) can be isolated using selective culture conditions, and cloned by limiting dilution. Cells which produce antibodies with the desired specificity can be selected by a suitable assay (e.g., ELISA).

[0054] Other suitable methods of producing or isolating antibodies of the requisite specificity can be used, including, for example, methods which select recombinant antibody from a library (e.g., a phage display library). Transgenic animals capable of producing a repertoire of human antibodies (e.g., XenoMouseTM (Abgenix, Fremont, CA)) can be produced using suitable methods (see, e.g., WO 98/24893 (Abgenix), published June 11, 1998 ; Kucherlapati, R. and Jakobovits, A.,

U.S. Patent No. 5,939,598; Jakobovits et al., Proc. Natl. Acad. Sci. USA, 90: 2551-2555 (1993); Jakobovits et al., Nature, 362: 255-258 (1993)). Additional methods for production of transgenic animals capable of producing a repertoire of human antibodies have been described (e.g., Lonberg et al., U.S. Patent No. 5,545,806 ; Surani et al., U. S. Patent No. 5,545,807; Lonberg et al., W097/13852).

[0055] In one embodiment, the method of inhibiting (reducing or preventing) graft rejection comprises administering an effective amount of a VEGF antagonist to an organ donor prior to donation. The antagonist may be an antibody and may be administered in combination with an immunosuppressive agent and/or a chemokine antagonist.

[0056] In another embodiment, the invention provides a method for inhibiting (reducing or preventing) graft rejection comprising administering to a graft recipient an effective amount of an antibody that inhibits VEGF and an effective amount of an immunosuppressive agent. Advantageously, the rejection-inhibiting effects of the antibody and immunosuppressive agents can be additive or synergistic, and can result in permanent engraftment. Preferably the above two embodiments are used in combination.

[0057] A further benefit of co-administration of a VEGF antagonist/anti-VEGF antibody and an immunosuppressive agent is that the dose of immunosuppressive agent required to inhibit graft rejection can be reduced to sub-therapeutic levels (e.g., a dose that does not inhibit graft rejection when administered as the sole therapeutic agent). The ability to reduce the dose of the immunosuppressive agent can greatly benefit the graft recipient as many immunosuppressive agents have severe and well-known side effects including, for example, increased incidence of infection, increased incidence of certain malignancies, diabetes mellitus, neurotoxicity, nephrotoxicity, hyperlipidemia, hypertension, hirsutism, gingival hyperplasia, impaired wound healing, lymphopenia, jaundice, anemia, alopecia and thrombocytopenia (Spencer, C. M., et al., Drugs, 54 (6) : 925-975 (1997); Physicians Desk Reference, 53d Edition, Medical Economics Co., pp. 2081-2082 (1999)).

[0058] The term "immunosuppressive agent", as used herein, refers to compounds which can inhibit an immune response. The immunosuppressive agent

used in the invention can be a novel compound or can be selected from the compounds which are known in the art, for example, MMF (Cellcept®), myfortic (mycophenolate sodium, Novartis), calcineurin inhibitors (e.g., cyclosporin A, FK-506), IL-2 signal transduction inhibitors (e.g., rapamycin), glucocorticoids (e.g., prednisone, dexamethasone, methylprednisolone, prednisolone), nucleic acid synthesis inhibitors (e.g., azathioprine, mercaptopurine, mycophenolic acid) and antibodies to lymphocytes or antigen-binding fragments thereof (e.g., OKT3, anti-IL2 receptor). Novel immunosuppressive agents can be identified by those of skill in the art using suitable methods, for example, screening compounds for the capacity to inhibit antigen-dependent T cell activation.

[0059] The immunosuppressive agent used is preferably a calcineurin inhibitor. More preferably the immunosuppressive agent used for co-therapy is cyclosporin A, MMF, rapomycin or/and steroids.

[0060] A "subject" or "animal" or "host" is preferably a human, but can also be a mammal in need of veterinary treatment, e.g., domestic animals (e.g., dogs, cats, and the like), farm animals (e.g., cows, sheep, fowl, pigs, horses, and the like) and laboratory animals (e.g., rats, mice, guinea pigs, and the like).

[0061] When co-administration of an antagonist and an additional therapeutic agent (immunosuppressive agent and/or chemokine inhibitor) is indicated or desired for inhibiting graft rejection, the antagonist can be administered before, concurrently with or after administration of the additional therapeutic agent. When the antagonist of and additional therapeutic agent are administered at different times, they are preferably administered within a suitable time period to provide substantial overlap of the pharmacological activity of the agents. The skilled artisan will be able to determine the appropriate timing for co-administration of an antagonist and an additional therapeutic agent depending on the particular agents selected and other factors.

[0062] An "effective amount" of a VEGF antagonist is an amount sufficient to achieve a desired therapeutic and/or prophylactic effect, such as an amount sufficient to inhibit graft rejection.

[0063] The term VEGF antagonists includes agents that decrease, inhibit, block or interfere with VEGF function including VEGF binding to the VEGF receptor KDR (VEGFR-2), VEGF receptor signaling and VEGF production.

[0064] The amount of agent administered to the individual will depend on the characteristics of the individual, such as general health, age, sex, body weight and tolerance to drugs as well as the degree, severity and type of rejection. The skilled artisan will be able to determine appropriate dosages depending on these and other factors. Typically, an effective amount can range from about 0. 1 mg per day to about 100 mg per day for an adult. Preferably, the dosage ranges from about 1 mg per day to about 100 mg per day.

[0065] Antibodies and antigen-binding fragments thereof, particularly human, humanized and chimeric antibodies and antigen-binding fragments can often be administered less frequently than other types of therapeutics. For example, an effective amount of such an antibody can range from about 0. 01 mg/kg to about 5 or 10 mg/kg administered daily, weekly, biweekly, monthly or less frequently.

[0066] The therapeutic agents can be administered by any suitable route, including, for example, orally (e.g., in capsules, suspensions or tablets) or by parenteral administration. Parenteral administration can include, for example, intramuscular, intravenous, intraarticular, intraarterial, intrathecal, subcutaneous, or intraperitoneal administration. The agent can also be administered orally , transdermally, topically, by inhalation (e.g., intrabronchial, intranasal, oral inhalation or intranasal drops) or rectally. Administration can be local or systemic as indicated. The preferred mode of administration can vary depending upon the particular agent chosen, however, oral or parenteral administration is generally preferred.

[0067] The agent can be administered as a neutral compound or as a salt. Salts of compounds containing an amine or other basic group can be obtained, for example, by reacting with a suitable organic or inorganic acid, such as hydrogen chloride, hydrogen bromide, acetic acid, perchloric acid and the like. Compounds with a quaternary ammonium group also contain a counteranion such as chloride, bromide, iodide, acetate, perchlorate and the like. Salts of compounds containing a carboxylic acid or other acidic functional group can be prepared by reacting with a

suitable base, for example, a hydroxide base. Salts of acidic functional groups contain a countercation such as sodium, potassium and the like.

[0068] Pharmaceutical compositions for co-therapy can comprise a VEGF antagonist and one or more additional therapeutic agents. An antagonist and an additional therapeutic agent can be components of separate pharmaceutical compositions which can be mixed together prior to administration or administered separately. Formulation will vary according to the route of administration selected (e.g., solution, emulsion, capsule). Suitable pharmaceutical or physiological carriers can contain inert ingredients which do not interact with the antagonist and/or additional therapeutic agent. Standard pharmaceutical formulation techniques can be employed, such as those described in Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, PA.

[0069] Suitable carriers for parenteral administration include, for example, sterile water, physiological saline, bacteriostatic saline (saline containing about 0. 9% benzyl alcohol), phosphate-buffered saline, Hank's solution, Ringer's-lactate and the like.

[0070] Methods for encapsulating compositions (such as in a coating of hard gelatin or cyclodextran) are known in the art (Baker, et al., "Controlled Release of Biological Active Agents", John Wiley and Sons, 1986).

[0071] The references cited throughout this application are herein incorporated by reference.

[0072] The documents mentioned herein are incorporated herein by reference.

[0073] It is understood that the foregoing detailed description and the following examples are illustrative only and are not to be taken as limitations upon the scope of the invention. Various changes and modifications to the disclosed embodiments, which will be apparent to those skilled in the art, may be made without departing from the spirit and scope of the present invention. Further, all patents, patent applications and publications cited herein are incorporated herein by reference.

EXAMPLES

Example 1

Methods

[0074] The following antibodies were used for immunohistochemistry in these studies: anti-mouse CD3, CD4, CD8, CD45 and CD31 (BD Pharmingen, San Diego, CA), anti-mouse IP-10 (Antigenix America Inc. Huntington Station, NY), Pan Macrophage Marker (Biosource International, Camarillo, CA), anti-human CD3, CD68, vWF (Dako, Carpenteria, CA) and anti-human VEGF (Santa Cruz, Biotechnology Inc., CA). Neutralizing monoclonal anti-human VEGF for *in vivo* studies was a gift of Genentech Inc., South San Francisco, CA (26;27); and neutralizing monoclonal hamster anti-murine IP-10 for *in vivo* studies was generated in Dr Andrew Luster's laboratory as described (28). The recombinant human cytokines interferon-γ and human VEGF165 were purchased from R&D Systems, Minneapolis, MN. Human IgG was purchased from Sigma Chemical Co, St Louis, MO and cyclosporine was purchased from Novartis (Basel, Switzerland). Endostatin (29-31) was a gift of Dr's Kashi Javaherian and Judah Folkman of Children's Hospital. Boston.

Generation of anti-VEGF antiserum

[0075] Anti-murine VEGF antiserum was prepared by Maine Biotechnology Services (Portland, ME) according to the methods of Tilton et al (32). We used the N-terminal sequence of secreted VEGF: CAPTTEGEQKSHEVIKFMDVYQRSY (SEQ ID NO: 1) coupled with keyhole limpet hemocyanin (KLH) using the maleimidobenzyl-N-hydroxylsuccinimide ester (MBS) crosslinker. New Zealand White rabbits were then immunized with 500 μg of peptide in Complete Freunds adjuvant by subcutaneous injection. Rabbits received further immunizations of 250 μg peptide in incomplete freunds adjuvant every 3 weeks. After 6 weeks, anti-VEGF titers were tested in standard ELISA. High titers of anti-VEGF were present in anti-sera up to a dilution of 1:156,000. Neutralizing activity was assessed in vitro by evaluating the ability of the antiserum to inhibit murine VEGF-induced proliferation of HUVEC as described (12). VEGF neutralizing activity of the anti-serum was also assessed *in vivo* using a modified version of a standard VEGF-induced angiogenesis assay (33). Briefly, Chinese

Hamster Ovary (CHO) cells (1 x 10^5 cells) expressing VEGF, as described (34) were injected into the ears of nude mice. The mice received control serum or anti-VEGF antiserum (0.8mls) intraperitoneally (i.p.) daily starting at day –1. Marked angiogenesis was evident at days 2 to 4 in control serum-treated mice, but not in mice that received anti-VEGF anti-serum (Figure 1). Overall, using this *in vivo* assay, there was ~80% inhibition of VEGF-induced angiogenesis by the anti-serum.

Animals

[0076] CB.17 SCID, C57BL/6 (H-2b) and BALB/c (H-2d) mice were purchased from Taconic Laboratory Animals and Services (Germantown, NY) and were used at age 6-8 weeks. IP-10 knockout (-/-) mice were generated in Dr Andrew Luster's laboratory as described (35).

HuSCID model

[0077] Full thickness human neonatal foreskin grafts were transplanted onto CB.17 SCID mice as described (4;36) and were allowed to heal for 4-6 weeks. For humanization, peripheral blood mononuclear cells (PBL) were isolated from leukopheresis packs obtained from the Blood Donor Centers at the Children's Hospital and the Dana Farber Cancer Institute, Boston, Massachusetts. All animals received 100 μL of anti asialo GM1 24 hours prior to the transfer of 3 x 10^8 PBL by i.p. injection. Anti-human VEGF antibody (Genentech) was administered on day –2, at the time of humanization and every other day (5 mg/kg i.p. in 100 μl saline as described (26;27). Control mice received 100 μl saline or human IgG. Animals were sacrificed and skin grafts were harvested after 7 or 14 days. All protocols were in accordance with the Animal Care and Use Committee, Children's Hospital, Boston, Massachusetts.

Immunohistochemistry

[0078] Frozen specimens were fixed in acetone, and formalin-fixed specimens were deparaffined. Immunohistochemistry was performed with primary and secondary horse-raddish peroxidase (HRP)-conjugated antibodies on frozen sections as described (4;12); on paraffin-embedded sections using the VectaStain Kit (Vector, Burlinghame, CA) and the Tyramide Signal Amplification (TSA) Biotin

system (NEN Life Science Products, Boston, MA) according to the manufacturers instructions. All specimens were developed in 3-amino ethylcarbazole (4;12) and were counterstained in Gill's hematoxylin.

Grid Counting

[0079] A standard calibrated grid method was used to quantitatively compare the amount of cellular infiltrate in skin grafts as described (36). Leukocytes were quantitated by the number of counting immunopositive cells within a calibrated grid at 400x magnification. Four to six adjacent non-overlapping fields of each specimen were analyzed in a blinded manner; and mean counts calculated.

Murine Cardiac Transplantation

[0080] BALB/c (H-2d) mice were used as recipients of fully mismatched C57BL/6 (H-2b) or IP-10-/- (H-2b) donor hearts. Vascularized intraabdominal heterotopic heart transplantation was performed as described (37). Recipients received intraperitoneal injections of either: a) 0.8ml of neutralizing anti-murine VEGF anti-serum (38);or control rabbit serum i.p. on days –1, 0, 2, 4 and 6, b) neutralizing anti-IP-10 (200□g) (39) i.p. on days –1, 1, 3, 5, and 7 ; or c) endostatin 20mg/kg/day (30) subcutaneously from day 0 to day 7 as determined by each experiment. Donor hearts were either harvested after 7 days as indicated, or were followed for the development of rejection. Harvested grafts were divided into three and were either snap frozen in liquid nitrogen or fixed in formalin for later analysis. Spleen cells obtained from recipients 7 days after transplantation were used in ELISPOT assays as described (40). Briefly, responder cells from controls or anti-VEGF-treated recipients were cocultured with stimulator donor spleen cells in a MLR for 48 hrs. ELISPOT for IFN-γ (Pharmingen, San Diego, CA) was performed to assess priming to alloantigen.

Cell culture

[0081] HUVEC were isolated from umbilical cords and were cultured as previously described (41). Murine endothelial cells were isolated from mouse heart by collagenase digestion and by subsequent sorting using magnetic beads (anti-CD31 and anti-ICAM-2) as described (42). The purity of endothelial cell cultures was

determined by the assessment of baseline cell surface expression of vWF and CD31 and by the cytokine-inducible expression of E-selectin. Peripheral blood leukocytes or mouse splenocytes were isolated by Ficoll Hypaque gradient centrifugation from blood obtained from healthy volunteers and mixed lymphocyte reactions (human, 2x 10^5 cells/well; or mouse, 4x 10^5 cells/well) were performed according to standard procedures (43). Proliferation was assessed after 3 days (mouse) or 5 days (human) by [H3] thymidine (1 μCi/ml) incorporation for the last 18hrs of the coculture. Reagents or controls were added to cultures as indicated and ELISA was performed for analysis of IFN-γ (Endogen Woburn, MA), IL-2 or IP-10 (R&D Systems, Minneapolis, MN) in coculture supernatants according to the manufacturer's instructions.

RT-PCR and RNase Protection Assays

[0082] RNA isolated from cultured HUVEC, skin and heart samples, using the Ultraspec RNA Isolation System (Biotecx Laboratories, Houston, TX), was reverse transcribed and PCR was performed using standard techniques (12). Sequence-specific primers for PCR were human VEGF (sense primer 5'TCACCGCCTCGGCTTGTCACA-3' (SEQ ID NO:3), antisense primer 5'-ATGAACTTTCTGCTGTCTTGG-3' (SEQ ID NO:3)) and β-actin (Stratagene, La Jolla, CA) was used as an internal control. PCR reactions were performed under the following conditions: 1 cycle at 94 °C for 5 minutes followed by 35 cycles at 94 °C for 1 minute, 60 °C for 1 minute, and 72 °C for 1 minute. The last cycle was extended to 7 minutes at 72 °C. The amplified products were resolved by electrophoresis in an ethidium bromide-stained 1.5% agarose gel.

[0083] RNase Protection assays was performed using the RiboQuant multiprobe template system (Pharmingen San Diego, CA), according to the manufacturers instructions (12). Relative signals were detected by autoradiography with Kodak MR film; and expression was quantified by densitometry by means of an AlphaImager 2000 system (Alpha Innotech, San Leandro, CA). For quantification, signals were standardized to the internal housekeeping gene GAPDH.

Statistical Analysis

[0084] Data were compared by nonparametric analysis using the Log Rank Test for allograft survival studies; and the Mann-Whitney test was used for immunohistological studies. P values less than 0.05 were considered statistically significant.

Results

[0085] Function of VEGF in allogeneic human leukocyte recruitment. We first used the huSCID mouse to determine the effect of VEGF on leukocyte trafficking into human skin. This model is unique as circulating human leukocytes in the huSCID selectively interact with human endothelial cells in the skin allograft (4;36), allowing for the analysis of molecules involved in endothelial cell-dependent mechanisms of human leukocyte trafficking. In our model, human foreskin is transplanted onto SCID mice and is allowed to heal for 4-6 weeks. Following adoptive transfer of human PBL into the mouse by intraperitoneal injection, CD4+ and CD8+ T cells as well as CD68+ monocytes/macrophages infiltrate the human skin over 7 to 14 days (4). As previously described (36), healed human skin in the huSCID is vascularized by both human and mouse vasculature, but human alloreactive leukocytes selectively interact and only mediate vasculitis of the human vessels. Thus, skin allografts do not slough in this model since the murine vasculature maintains blood flow and integrity of the skin despite marked leukocyte infiltration and marked vasculitis of the human vessels (4;44).

[0086] We initially evaluated the expression of VEGF in non-infiltrated and infiltrated human skin specimens from non humanized or huSCID mice respectively, and found enhanced VEGF mRNA and protein expression in association with infiltrates (Figure 2). By immunohistochemistry, VEGF protein was expressed by both inflammatory infiltrates and by vascular endothelial cells (Figure 2 d-e). In contrast, in skin specimens without infiltrates, VEGF was only found on keratinocytes and endothelial cells with minimal expression overall (Figure 2 f). We treated huSCID mice with a neutralizing anti-human VEGF, human IgG or saline. Consistent with its known function as a monocyte chemoattractant (11) anti-VEGF had a significant inhibitory effect on the recruitment of CD68+ monocyte/macrophages (Figure 3). Few CD68+ cells were evident in skins harvested from animals treated

with anti-VEGF (Figure 3 i). Moreover, unexpectedly we found that anti-VEGF was most potent to inhibit CD3+ T cell trafficking into human skin (Figure 3 d-f). By quantitative grid counting analysis, there was a statistically significant decrease in CD3+ T cell infiltrates in anti-VEGF treated huSCID animals both at day 7 and at day 14 ($P<0.003$ and $P<0.004$, Figure 3 j) respectively. Therefore, VEGF is functional for peripheral recruitment and for the trafficking of human allogeneic lymphocytes and monocytes *in vivo*.

[0087] We also assessed the effect of anti-VEGF on the angiogenesis reaction, which we have previously reported to be associated with mononuclear cell infiltration in this model (4). Our findings were that anti-VEGF markedly inhibited angiogenesis (data not shown), which is consistent with the interpretation that locally expressed VEGF is also of functional importance in leukocyte-associated angiogenesis.

In vitro analysis of VEGF-dependent chemokine production in endothelial cells

[0088] The function of VEGF as a monocyte chemoattractant is established in vitro (11). However, our finding that VEGF promotes T cell recruitment has not been previously reported in vitro or *in vivo*. To study the mechanism of function of VEGF on T cell recruitment, we next assessed the effect of VEGF on human endothelial cell activation in vitro. Treatment of confluent cultures of endothelial cells with increasing concentrations of VEGF resulted in a dose dependent increase in the expression of the endothelial cell adhesion molecules E-selectin, ICAM-1 and VCAM-1 (not shown, and as reported by others (9;10)). In addition, following treatment with VEGF, we found that the endothelial cell expression of the chemokines monocyte chemoattractant protein (MCP)-1 and interleukin-8 (IL-8) is also increased (Figure 4 a). MCP-1 peaked in expression between 1 and 5 hrs following treatment; and expression persisted for up to 24 hrs (Figure 4 b). In contrast, IL-8 peaked in expression at approximately 2 hrs following treatment, and decreased in expression by 24hrs. But, there was no increase in the expression of T cell chemoattractant chemokines lymphotactin, RANTES, IFN-inducible protein-10 (IP-10) (Figure 4) or Mig (not shown) after VEGF treatment.

[0089] Since IFN-γ-induced chemokines play a major role in the local recruitment of allogeneic lymphocytes, we also determined an effect of VEGF on IFN-γ-mediated activation of the potent T cell chemoattractant chemokines IP-10 and Mig. We treated endothelial cells with IFN-γ and found induced expression of IP-10 and Mig as reported (45;46). Moreover, we observed that a combination of VEGF with IFN-γ resulted in a synergistic induction of endothelial cell IP-10 mRNA and protein expression, but not Mig expression (Figure 4 c-g and not shown). In addition, we observed that IFN-γ inhibited the expression of IL-8, and that combinations of VEGF and IFN-γ restored IL-8 expression (Figure 4 c and d). Thus, VEGF may promote human allogeneic lymphocyte trafficking *in vivo* via regulation of endothelial cell production of chemokines including the potent T cell chemokine IP-10.

Blockade of VEGF in a fully mismatched murine model of acute cardiac allograft rejection

[0090] Our results also raise the possibility that VEGF is of importance in allograft rejection. Allograft rejection is a complex multicellular process that is critically dependent upon T cell recognition of alloantigen processed and presented to T cells by either donor or recipient antigen presenting cells (47). The subsequent recruitment of activated T cells and effector cells into an allograft has been shown to be mediated in part by locally expressed leukocyte-endothelial adhesion molecules (48) and chemokines (49). We used a physiological *in vivo* model of acute allograft rejection in which fully MHC mismatched C57BL/6 ($H\text{-}2^b$) donor hearts were transplanted into BALB/c ($H\text{-}2^d$) recipients. Similar to our findings in the huSCID (above), VEGF was expressed in association with graft infiltrates and acute rejection (Figure 5 a-b). Administration of our neutralizing anti-VEGF antiserum resulted in a significant prolongation of allograft survival up to 25 days post-transplant vs. control serum treated animals (Figure 5 c, $P<0.01$). Severe cellular rejection with extensive mononuclear cell infiltration and myocyte necrosis was evident in sections of day 7 rejecting grafts in recipients treated with control rabbit serum (Figure 5 d-g). In contrast, we observed only a mild to moderate infiltrate, reduced numbers of CD3+ T cells and monocyte/macrophages, less myocyte damage and minimal vasculitis in allografts from recipients treated with anti-VEGF anti-serum (Figures 5 I-L). Moreover, the T cell chemoattractant IP-10 was observed in abundance on multiple

cell types in rejecting grafts (Figure 5 H), but there was minimal expression in grafts harvested from animals treated with anti-VEGF (Figure 5 m). Therefore, the intense expression of VEGF in allografts *in vivo* is of mechanistic significance to promote the development of rejection.

Mechanism of function of VEGF in allograft rejection in vivo

[0091] The effect of anti-VEGF on leukocyte trafficking and allograft rejection in our *in vivo* studies could be explained by an effect of VEGF on T cell activation responses. However, addition of our anti-VEGF antibodies to cocultures in human or mouse mixed lymphocyte reactions (MLRs) failed to inhibit T cell activation responses (Figure 6 a-b). Also, the *in vivo* priming of T cells to alloantigen as manifested by ELISPOT analysis of IFN-γ in murine recipients of allografts (above) was unaltered by anti-VEGF treatment (Figure 6 c). Thus, VEGF does not function in alloimmune T cell activation in vitro or *in vivo*.

[0092] We next assessed whether the intense intragraft expression of VEGF in acute rejection could mediate leukocyte recruitment in part via neovascularization. By immunohistochemistry, we found that endothelial CD31 was expressed diffusely on large vessels as well as microvessels throughout all grafts examined. (Figure 7A). Moreover, the staining pattern of CD31 as well as the vascularization of isografts was very similar to that found in anti-VEGF-treated grafts. In contrast, we found fewer CD31-expressing vessels in control-Ig-treated rejecting grafts; and some focal areas of rejecting grafts were devoid of any vascular staining (Figure 7A, and not shown). To assess a role for angiogenesis in acute rejection, we also treated animals with endostatin, a known endothelial cell-specific inhibitor of angiogenesis (29) (Figures 7B – 7D). Our findings were that endostatin had a minimal effect on allograft survival, prolonging survival in 3 out of 8 animals; and failed to have any effect on leukocyte trafficking into the graft (Figures 7B – 7D). Since the effect of endostatin was so markedly different than that following treatment with anti-VEGF (Figure 5), we interpret these data to suggest that VEGF mediates leukocyte trafficking into an allograft independent of an effect on angiogenesis.

[0093] To further define the role of VEGF in T cell recruitment into cardiac allografts, we examined the effect of VEGF in primary cultures murine

myocardial endothelial cells in vitro. In contrast to our findings in human EC, we found that VEGF alone induced the expression of IP-10 in murine EC (Figure 8 a). Thus, it is possible that VEGF may regulate T cell trafficking primarily via IP-10-dependent mechanisms. Indeed, local intragraft endothelial cell expression of IP-10 has been previously reported to be of critical importance in the recruitment of T cells into allografts in rejection (39). Transplanted animals were next treated with neutralizing anti-IP-10 antibodies alone or in combination with anti-VEGF (Figure 8 B). Our findings were that anti-IP-10 alone prolonged graft survival; and that combined treatment with anti-IP-10 and anti-VEGF significantly prolonged allograft survival vs. treatment with anti-IP-10 alone or anti-VEGF alone (Figure 8B, $P< 0.005$ and $P< 0.03$ respectively). This is suggestive that a major effect of anti-VEGF *in vivo* is independent of IP-10. To further confirm this interpretation, we also transplanted allografts from IP-10 knockout (-/-) donors into wild type mice and again found that anti-VEGF prolonged survival in the absence of local IP-10 production (Figure 8C, $P<0.04$).

[0094] Lastly, we examined adhesion molecule and chemokine expression *in vivo* in day 7 cardiac allografts harvested from control or anti-VEGF treated wild-type mice. By RNase protection, we found a significant decrease in the expression of E-selectin, ICAM-1 and VCAM-1 in anti-VEGF-treated animals as compared to controls (Figure 9A). In addition, anti-VEGF markedly inhibited the intragraft expression of several T cell chemoattractant chemokines (including Lymphotactin, Rantes and IP-10) as well as the monocyte chemoattractant MCP-1 (Figure 9 b). Thus, blockade of VEGF *in vivo* inhibits the local expression of several chemokines that are not directly regulated by VEGF. We interpret our findings to suggest that VEGF is of importance in allograft rejection at early times post transplantation and prior to the induced expression and function of T cell-derived chemokines. Intragraft VEGF expression appears to promote early T cell and monocyte trafficking; and that subsequent activation events result in additional VEGF expression as well as the expression of chemokines (including IP-10) both of which are additive to promote rejection.

Discussion

[0095] VEGF is a most potent and critical mediator of physiological as well as pathological angiogenesis. Its expression and function have been reported in several chronic inflammatory processes, however its mechanism of function in immunity has been unclear. Recent reports have defined the angiogenesis reaction mediated by VEGF to be proinflammatory in chronic inflammation (7). Here, we provide evidence that VEGF promotes endothelial cell chemokine production in vitro and *in vivo*, and functions in the recruitment of monocytes and T cells into allografts. VEGF is thus an important proinflammatory cytokine in transplant rejection.

[0096] The expression of VEGF has been reported to be associated with allograft rejection (especially chronic rejection) in both experimental models and following human transplantation (22;24;25). In addition, it has been reported that transplant recipient genotypes encoding high VEGF production are associated with increased risk for the development of acute renal allograft rejection (23). Here, we extend upon these observations and demonstrate that intragraft VEGF has a biological effect *in vivo* to promote rejection, as leukocyte infiltration is inhibited following treatment with neutralizing anti-VEGF. Moreover, we demonstrate that induced intragraft VEGF is unlikely to be functional for the development of angiogenesis, as neovascularization is not a component of the rejection process. Rather, we show that the mechanism of function of VEGF appears to involve its ability to mediate intragraft leukocyte trafficking.

[0097] It is well established that the process of recruitment involves coordinate interactions among adhesion molecules and chemokines (3;49). Multiple individual molecules have been shown to be functional in the process of rejection (49-52), suggesting that there is some redundancy in the function of adhesion molecules and chemokines *in vivo*. It is known that VEGF induces the expression of adhesion molecules in human endothelial cells (9;10) and two recent reports have pointed to an effect of VEGF on chemokine production (53;54). Here, we extend upon these observations and show that VEGF regulates IP-10, MCP-1 and IL-8; as well as the endothelial cell adhesion molecules E-selectin, ICAM-1 and VCAM-1 in vitro and *in vivo*. All of these molecules are expressed in association with rejection; and blockade of several molecules or their ligands individually (e.g. IP-10, MCP-1, ICAM-1, VCAM-1) (49-52) have been shown to prolong allograft survival in murine models.

Our data in this study imply that the ability of VEGF to regulate several of these molecules is the basis for its function in leukocyte trafficking. However, we have not fully addressed this complex question at an individual molecular level; and thus, this interpretation will require further study. Nevertheless, of all chemokine-chemokine receptor interactions studied to date, most profound effects are seen when the T cell chemoattractant IP-10 or its ligand CXCR3 are targeted (39;50;55). We find that VEGF-dependent T cell trafficking and rejection *in vivo* is not restricted to IP-10-dependent trafficking. Our results therefore provide a framework for an analysis of the role of VEGF in the expression and function of individual chemokines and adhesion molecules. Chemokine and adhesion molecule knockout mice are available for future studies which we believe will define a more general mechanism by which VEGF is of importance in leukocyte trafficking, in rejection and in other cell-mediated immune inflammatory diseases (7;38).

[0098] Another interpretation of our findings is that the major proinflammatory function of VEGF is in the initiation of the inflammatory cascade, rather than in subsequent events resulting from T cell-mediated activation responses. Consistent with this possibility, it is important to note that hypoxia is the major stimulus for VEGF expression (16). This suggests that VEGF will be produced locally within allografts immediately following transplantation. Additional insults including platelet and leukocyte recruitment into the graft will further facilitate the expression of local VEGF (12) as well as other cytokines and chemokines (56) that are of importance in the rejection process (47;49;57). Here, we provide evidence that early VEGF expression promotes T cell and monocyte recruitment. We suggest that the subsequent induced expression of chemokines (e.g. IP-10) and VEGF are additive to promote the development of rejection. Since it is well established that early insults to allografts (such as prolonged ischemia) have long term consequences (58), dysregulation of VEGF at early times following transplantation may have beneficial effects to promote graft survival. Indeed, in support of this possibility it has been noted that early (but not late) blockade of VEGF can limit the development of chronic arthritis (38).

[0099] In summary, our results for the first time provide compelling evidence that VEGF is a proinflammatory cytokine in allograft rejection. In addition,

they provide insight into the role of VEGF in cell-mediated inflammatory reactions in immunity.

Example 2

[00100] Graft survival is also significantly prolonged when anti-VEGF is combined with the immunosuppressant CsA[30]. We found that treatment with both anti-VEGF and CsA significantly prolonged allogaft survival as compared to CsA alone ($P< 0.001$) or anti-VEGF alone ($P< 0.03$). This suggests that blockade of VEGF may have clinical utility in the future, as T cell-directed immunosuppressants are currently the mainstay of therapy following human transplantation. Also, mechanistically these data are consistent with our observations that CsA enhances VEGF responses in human endothelial cells and thus that VEGF mediated proinflammatory events may be more prominent when CsA is used *in vivo*. Our observation is also consistent with the possibility that VEGF alone has no significant effects on alloimmune T cell activation responses *in vivo* and that the major effect of anti-VEGF is via dysregulation of local endothelial cell chemoattractants that facilitate T cell recruitment.

Example 3

[00101] Development of a blocking monoclonal antibody to VEGF: Rat anti-mouse VEGF monoclonal antibodies were generated using the the N-terminal sequence of secreted VEGF: CAPTTEGEQKSHEVIKFMDVYQRSY coupled with keyhole limpet hemocyanin (KLH) using the maleimidobenzyl-N-hydroxylsuccinimide ester (MBS) crosslinker. Fischer and Noble rats were immunized and hybridoma fusion products generated according to standard protocols. Fusion products were then chosen for scale up based their anti-VEGF OD reading in ELISA. The fusion from 300326 accession (Fisher rats) yielded 53 fusion products and the fusion from 300329 accession (Noble rats) yielded 15 fusion products. All chosen fusion products were scaled up, cryopreserved and screened again to confirm antibody production and the class of the specific antibody if present. The results of the secondary screens of the Noble rats demonstrated that there were 6 positives out

15 hopefuls, and 3 out of those 6 were strong IgG's. These were subcloned and scaled up and tested for VEGF neutralizing potential. We found that one lead antibody, called 2G11 had VEGF blocking ability in an in vitro endothelial cell proliferation assay.

[00102] Anti-VEGFmAB 2G11 neutralizes VEGF in vivo. VEGF neutralizing activity of 2G11 was assessed in vivo using a modified version of a standard VEGF-induced angiogenesis assay. Briefly, Chinese Hamster Ovary (CHO) cells (1×10^5 cells) expressing VEGF were injected into the ears of nude mice. This results in a most potent and aggressive angiogenesis reaction. The mice received either no treatment or 2G11 anti-VEGF in increasing doses i.p. daily starting at day – 1. Illustrated in Figure 11 A, is seen marked angiogenesis in response to CHO-VEGF cell injection at day 2, whereas in Figure 11 B, the antibody at a dose of 200mcg/day inhibits this CHO-VEGF induced angiogenesis reaction. This clearly indicates that 2G11 blocks VEGF function in vivo. (Same result is seen at day 4, not shown).

Example 4

[00103] ROLE OF VEGF-VEGF-R SIGNALING IN ISCHEMIA REPERFUSION INJURY.

Design:

[00104] To evaluate the effect of Vascular Endothelial Growth Factor (VEGF) on ischemia reperfusion injury, normal mice were treated either with (1) anti-VEGF antibody, (2) PTK787, (a VEGF receptor signaling inhibitor, Novartis Pharma, MTA with Children's Hospital) or (3) Mycophenolate Mofetil (MMF). Our in vitro studies demonstrated that MMF weakly inhibits post-VEGF receptor signaling. We also evaluated the effect of MMF in combination with anti-VEGF or PTK787.

Kidney Model:

[00105] Unilateral acute tubular necrosis (ATN) in mice by clamping of the renal pedicle to produce ischemia. These procedures are performed under general anesthesia. Ischemic injury occurs using different timed protocols (30 mins for studies illustrated in Figure 12), after which the clamps are removed. Animals are monitored

daily. Tissue is harvested at various time points after surgery for up to 4 weeks. Graft function is monitored by measurement of serum creatinine every few hrs for first 24 hrs, then daily or every other day. Treatment involves the use of PTK 787 at a dose of 4 mg/day and mycophenolate moefitil (MMF) at 2 mg/day.

[00106] Mice (n= 4-10 per time point/group) were untreated or treated with PTK787 at a dose of 100 mg/kg/day divided twice a day (for a total of 2 mg/mouse twice a day, total dose 4 mg). The total dose for MMF was 100 mg/kg once daily (for a total dose of MMF at 2 mg/mouse/day.) Mice were treated intraperitoneally, starting on Day –1 of the experiment. The doses used for both PTK787 and MMF were based on the pilot in vivo experiments demonstrating that these concentrations inhibit VEGF-induced angiogenesis in vivo.

Results:

[00107] Untreated mice develop acute renal failure, manifested by an increase in serum creatinine by 24 hours following ischemia. The renal failure was temporary and recoverable, with serum creatinine returning to baseline between 2 and 6 days following the procedure. The transient nature of this acute renal failure enables assessment of protection with the agents described above. As illustrated in the figure 12, treatment of the mice with PTK787 protected the animals from a rise in serum creatinine and acute renal failure. In addition, treatment of animals with MMF somewhat reduced the degree of renal failure (with 1 out of 5 animals having some degree of abnormal creatinine.) The combination of MMF and PTK was remarkably protective in this model.

Conclusion:

[00108] Pretreatment of animals with MMF or PTK787 alone or PTK787 in combination with MMF protects animals from acute renal failure, resulting from ischemia reperfusion injury. We have found that treatment of mice with a blocking anti-VEGF antibody also tends to reduce the degree of renal injury associated with ischemia reperfusion.

Liver Model:

[00109] We have also evaluated the effect of blocking anti-VEGF antibody in an established model of liver ischemia reperfusion injury. The liver model is described in the reference (Transplantation, 2003 April 27: 75; pages 1118-1123) and is similar to the kidney model described above. In this study, animals were treated with the anti-VEGF anti-serum (0.8 ml intraperitoneally) on Day –1 and at the time of ischemia. Control antibody treated animals developed a marked rise in serum transaminase levels (ALT) with ALT a mean level of 3,619 + 750. In contrast, anti-VEGF treated animals only developed a mild increase in transaminase levels of 413 + 126. (The rise in ALT transaminase is indicative of liver injury. Normal levels are less than 100.) Taken together, these data suggest that blockade of VEGF has protective effects in the prevention of ischemia reperfusion injury in both the kidney and the liver.

Implications:

[00110] Transplantation characteristically involves ischemia and reperfusion. All donor organs are harvested from a donor and suffer from different degrees of ischemia. The greater the ischemia, the worse the injury. Ischemia reperfusion is established to be detrimental to short- and long-term outcome. In the case of kidney donors, with existing kidney disease, such as occurs in older donors, the effect of ischemia is very notable and outcome following transplantation is much poorer as compared to healthy young donors. These kidney donors are referred to as "marginal donors." Due to the shortage of organ donors and ability for transplantation, the use of marginal donors is increasing nationally. In general, even for the most perfect of organs, it is thought that ischemia reperfusion initiates damage that progresses to limit the long-term survival of organ transplants. Thus, any treatment that can prevent ischemia reperfusion might have some benefit for the success of transplantation. Treatment of a donor with an agent, such as a VEGF antagonist prior to the harvest of the organ, will prevent ischemia reperfusion and thus prolong the survival of that organ in a patient. Our studies suggest that the donor and recipient of a transplanted organ could be treated for a limited period of time to prevent ongoing damage from ischemia reperfusion. The treatment period for the donor will be days up to one week. The treatment for the recipient will begin prior to

or on the day of the transplant and continue for about 30 days to about 90 days post-transplant.

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[00111] Although the foregoing invention has been described in some detail by way of illustration and example for the purposes of clarity of understanding, one skilled in the art will easily ascertain that certain changes and modifications may be practiced without departing from the spirit and scope of the appended claims.